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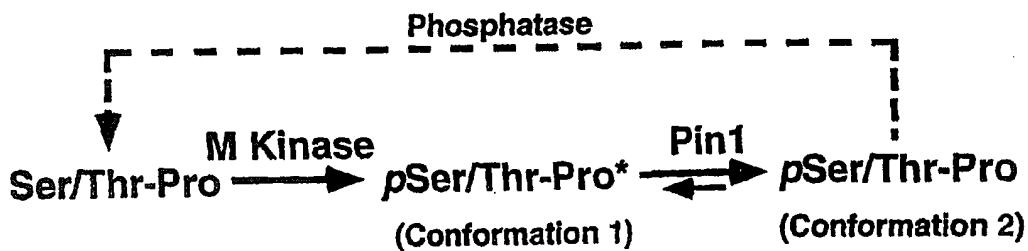
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(54) Title: INHIBITORS OF PHOSPHOSERINE AND PHOSPHOTHREONINE-PROLINE-SPECIFIC ISOMERASES



(57) Abstract

Peptides and peptide mimetics that inhibit phosphoserine- or phosphothreonine-specific pepidyl prolyl isomerases are described.

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INHIBITORS OF PHOSPHOSERINE
AND PHOSPHOTHREONINE-PROLINE-SPECIFIC ISOMERASES

BACKGROUND

Events of the eukaryotic cell cycle are regulated by an evolutionarily 5 conserved set of protein kinases. The cyclin-dependent kinases (Cdks) are important for driving cells through different phases of the cell cycle and their sequential activation and inactivation are tightly regulated. At the G2/M transition, activation of the mitotic Cdk, Cdc2, requires multiple events; these include the synthesis and binding of cyclin B, phosphorylation on Cdc2 at an activating site by Cak, and 10 finally, Cdc25-dependent dephosphorylation of inactivating sites that have been phosphorylated by Wee1 and Myt1 (P. Nurse, *Cell* 79:547 (1994); R. W. King, P. K. Jackson, M. W. Kirschner, *Cell* 79:563 (1994); T. R. Coleman, W. G. Dunphy, *Curr. Opin. Cell Biol.* 6:877 (1994)).

How activation of a Cdk elicits the downstream events of cell cycle 15 progression is less well understood. Activation of cyclin B/Cdc2 leads to the phosphorylation of a large number of proteins, mainly on sites containing a Ser/Thr-Pro motif. Protein phosphorylation is believed to alter the functions of proteins to trigger the events of mitosis. In a few cases, mitotic phosphorylation has been shown to regulate mitotic events (R. Heald and F. McKeon, *Cell* 61:579 (1990); E. 20 Bailly, et al., *Nature* 350:715 (1991); A. Blangy, et al., *Cell* 83:1159 (1995)). However, it is not understood how the rapid changes in mitotic phosphorylation are converted to the sequential events of mitosis.

An important experimental tool which has uncovered the general role of 25 phosphorylation in mitotic regulation is the MPM-2 monoclonal antibody (F. M. Davis, et al., *Proc. Natl. Acad. Sci. USA* 80:2926 (1983)). MPM-2 recognizes a Phospho.Ser/Thr-Pro epitope on approximately 50 proteins which are localized to various mitotic structures (J. M. Westendorf, P. N. Rao, L. Gerace, *Proc. Natl. Acad. Sci. USA* 91:714-8 (1994)). Several important mitotic regulators are recognized by this antibody, including Cdc25, Wee1, topoisomerase IIa, Cdc27, Map 4, INCENP 30 and NIMA (Stukenberg, P.T., K.D. Lustig, T.J. McGarry, R.W. King, J. Kuang and M.W. Kirschner, *Curr Biol* 7:338-348 (1997)).

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Currently six kinases have been shown to phosphorylate proteins *in vitro* to produce the MPM-2 epitope: Cdc2, Polo-like kinase (Plk1), NIMA, MAP kinase, a MAP kinase (MEK), and an unidentified activity ME-H (Kuang, J. and C.L. Ashorn., *J Cell Biol* 123:859-868 (1993); Taagepera *et al.*, *Mol Biol Cell* 5:1243-1251 (1994); Kumagai, A. and W.G. Dunphy, *Science* 273:1377-1380 (1996); Renzi, L., M.S. Gersch, M.S. Campbell, L. Wu, S.A. Osmani and G.J. Gorbsky, *J Cell Sci* 110:2013-2025 (1997)). However, these kinases also phosphorylate substrates that do not generate the MPM-2 epitope especially in cell cycle stages other than mitosis. This suggests that there are additional features that are required 10 for the recognition by MPM-2. Determination of the optimal MPM-2 binding sequence have confirmed the importance of amino acid residues flanking the Phospho Ser/Thr-Pro motif for the MPM-2 recognition (Westendorf, J.M., P.N. Rao and L. Gerace. *Proc Natl Acad Sci U S A* 91:714-718 (1994)). Westendorf, *et al.*, 15 1994).

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SUMMARY OF THE INVENTION

The present invention is based on the discovery that an essential mitotic peptidyl prolyl isomerase specifically recognizes phosphorylated serine/threonine-proline bonds present in mitotic phosphoproteins. As a result of this discovery, a 20 novel class of molecular compounds are available with activity to act as inhibitors of phosphoserine/phosphothreonine-proline specific peptidyl prolyl isomerases, in particular the peptidyl prolyl isomerase, Pin1, and other Pin1-like isomerases. Accordingly, these molecular inhibitors are useful to treat disorders of cell 25 proliferation such as hyperplastic or neoplastic disorders, wherein treatment of the disorder with an inhibitor of the present invention results in the arrest of mitosis and apoptosis (cell death) of the target cells.

The inhibitor compounds of the present invention include any molecule that binds into the active site of the phosphoserine- or phosphothreonine-proline specific peptidyl prolyl isomerase and, upon binding to the isomerase, inhibits the isomerase 30 activity. Encompassed by the present invention are inhibitor compounds that mimic the structure and conformation of the substrate moiety when bound to the catalytic site (also referred to herein as the active site) of the isomerase. Molecular inhibitors of the the present invention will typically have an inhibition constant (K_i) in the nanomolar to micromolar range. Specifically encompassed herein are organic

molecules that mimic the structure and conformation of pSer/pThr and bind to the isomerase of interest, thereby inhibiting its activity.

The inhibitor compounds of the present invention inculde inhibitors that comprise a core region that mimics the pSer/pThr-Pro peptide moiety of the phosphoserine- or phosphothreonine-proline peptidyl prolyl isomerase substrate. Encompassed by the present invention are inhibitors that comprise the pSer/pThr mimic moiety with the mimic moiety being flanked on one side by hydrophobic groups and on the other side by hydrophobic or positively charged groups, wherein the groups would contact the active site of the isomerase of interest.

10 The inhibitor compounds of the present invention include compounds that contain a core sequence comprising xSer/xThrY wherein "x" is a negatively charged tetra- or pentavalent moiety and "Y" is a Pro (proline) or a Pro analog. More specifically, the inhibitors of the present invention include compounds that inhibit a phosphoserine- or phosphothreonine-proline specific peptidyl-prolyl isomerase comprising a protein, polypeptide, peptide and/or a peptide mimetic wherein said protein, polypeptide, peptide or peptide mimetic comprises pSer/pThr. Specifically encompassed are inhibitors that have the core sequence of XXXpSer-pProXXX, wherein X is any L-amino acid or D-amino acid.

20 Candidate molecules of the present invention are evaluated for inhibitory activity in competitive inhibition assays. For example, the assay mixture would include the candidate molecule to be tested for inhibiting activity, the isomerase of interest and the intended substrate of the isomerase of interest. This admixture is maintained for a time sufficient and under conditions sufficient for the isomerase of interest to bind and catalyze the isomerization of its intended substrate. The catalytic 25 activity of the isomerase of interest in the presence of the candidate inhibitor is then compared with the activity of the isomerase in the absence of the candidate inhibitor. If the activity of the isomerase in the presence of the inhibitor is less than the activity of the isomerase in the absence of the inhibitor, the candidate inhibitor is suitable for use as an inhibitor of the isomerase of interest.

30 Encompassed by the present invention are inhibitors of interphase-specific pSer/pThr-Pro specific peptidyl prolyl isomerases. Specifically encompassed by the present invention are inhibitors of the essential mitotic peptidyl prolyl isomerase, Pin1, and other PIN1-like isomerases.

Also encompassed by the present invention are methods of inhibiting mitotic 35 peptidyl-prolyl isomerases comprising administering an effective amount of an

inhibitor as described herein. For example, a composition comprising an effective amount of the inhibitor and a pharmaceutically acceptable carrier can be administered to an individual in need thereof. Specifically encompassed are methods of inhibiting unwanted cell growth resulting from a hyperplastic or neoplastic disorder. Also encompassed by the present invention are methods of inhibiting cell growth in target cells, comprising contacting the cells with an inhibitor as described herein.

The present invention also relates to libraries of peptides that comprises a mixture of substantially equimolar amounts of peptides comprising the sequence 10 NH₂-MAXXpSXXXAKK, wherein for each peptide X is any amino acid.

The present invention also relates to methods of identifying a phosphorserine- or phosphothreonine-specific peptidyl prolyl isomerase inhibitor comprising the steps of:

- 15 a) providing a library of compounds that comprises a mixture of substantially equimolar amounts of peptides comprising the sequence X₁X₂X₃pS-PX₄X₅X₆, wherein for each peptide X is any amino acid;
- b) contacting the library of a) with the peptidyl prolyl isomerase of interest under binding conditions for time sufficient for the isomerase to bind to the peptides;
- 20 c) determining the amino acid sequences of peptides bound to the isomerase of interest;
- d) synthesizing the peptides of c); and
- e) assaying peptides of d) for *cis/trans* isomerization by the peptidyl prolyl isomerase of interest to determine which peptides undergo 25 isomerization by the isomerase of interest, thus identifying peptides that bind to the isomerase and are suitable for use as inhibitors of the isomerase of interest.

The present invention further relates to methods of identifying a phosphorserine or phosphothreonine-specific peptidyl prolyl isomerase inhibitor comprising the steps of:

- a) providing the peptidyl prolyl isomerase of interest;
- b) mixing the isomerase of interest with:
 - i) a candidate molecule and
 - ii) the substrate of the isomerase of interest

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- to form an admixture of the isomerase of interest, candidate molecule and substrate;
- 5 c) maintaining the admixture of b) under conditions sufficient for the isomerase of interest to catalyze the *cis/trans* isomerization of the substrate; and
- d) determining the K_i of the candidate molecule, wherein a K_i of 10 micromolar or less is indicative of an inhibitor of the peptidyl prolyl isomerase of interest.

BRIEF DESCRIPTION OF THE FIGURES

10 Figure 1 depicts a model for the Pin1-dependent regulation of mitosis-specific phosphoproteins that are phosphorylated by Cdc2 and other mitotic kinases (M kinase).

Figure 2 is a graphic representation of the results of an experiment showing that Pin1 inhibits mitotic division in *Xenopus* embryos.

15 Figures 3A-C are graphic representations showing that Pin1, but not the mutant, directly inhibits the ability of Cdc25 to activate cyclin B/Cdc2.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is related to the discovery that an essential mitotic peptidyl-prolyl isomerase (PPIase) recognizes phosphorylated serine/threonine (pSer/pThr) bonds present in mitotic phosphoproteins. Pin1 is an essential peptidyl-prolyl *cis-trans* isomerase (PPIase). It is distinct from two other well-characterized PPIase families: the cyclophilins and the FK-506-binding proteins (FKBPs), which are targets for the immunosuppressive drugs cyclosporin A and FK506, respectively (reviewed in Schreiber, S.L., *Science* 251:283-287 (1991)).

25 PPIases are ubiquitous enzymes that catalyze rotation about the peptide bond preceding a Pro residue, and may accelerate the folding and trafficking of some proteins (reviewed in Schmid, F.X., *Curr. Biol.* 5:993-994 (1995)). Interestingly, inhibition of PPIase activity is not required for the immunosuppressive property of cyclosporin A and FK506. Furthermore, neither the cyclophilins nor the FKBPs are 30 essential for normal cell growth. Thus, evidence for the biological importance of PPIase enzymatic activity has been limited.

In contrast, Pin1 contains a PPIase domain that is essential for cell cycle progression and its subcellular localization is tightly regulated at the G2/M transition

(Lu, K.P., S.D. Hanes and T. Hunter, *Nature* 380:544-547 (1996)). Pin1 is localized in a defined nuclear substructure in interphase, but is concentrated to the condensed chromatin, with some staining in other structures, during mitosis. Furthermore, depletion of Pin1 protein in HeLa cells or Pin1/Ess1p in yeast results in mitotic 5 arrest, whereas overexpression of Pin1 induces a G2 arrest. These results suggest that Pin1 is an essential mitotic regulator that both negatively regulates entry into mitosis and is required for progression through mitosis.

As described herein, Pin1-binding proteins have been identified in human cells and *Xenopus* extracts. Pin1 has been identified in all eukaryotic organisms 10 where examined, including plants, yeast, Aspergillus, and mammals (sequences deposited in GenBank). Results indicate that although Pin1 levels are constant throughout the cell cycle, the interaction of Pin1 and its targets is cell cycle-regulated and depends upon mitotic phosphorylation of target proteins.

Pin1 directly interacts with a large subset of mitosis-specific 15 phosphoproteins, which includes Cdc25, Wee1, Myt1, Plk1, Cdc27 and E-MAP115 as well as some others recently identified by a screen for mitotic phosphoproteins (Stukenberg, P.T., K.D. Lustig, T.J. McGarry, R.W. King, J. Kuang and M.W. Kirschne, *Curr Biol* 7:338-348 (1997)). Many of these Pin1-interacting proteins are also recognized by the MPM-2 antibody. In functional assays, microinjection of 20 Pin1 inhibits mitotic division in *Xenopus* embryos and entry into mitosis in *Xenopus* extracts, as is the case in HeLa and yeast cells. Furthermore, Pin1 binds the mitotically phosphorylated form of Cdc25 in vitro and in vivo, and it binds Cdc25 on the important phosphorylation sites and inhibits its activity. This characterization of the Pin1-Cdc25 interaction can at least partially explain the ability of Pin1 to inhibit 25 the G2/M transition. All these activities of Pin1 are dependent upon the ability of Pin1 to mitotic phosphoproteins since the activities are disrupted by point mutations which inhibit the ability of Pin1 to recognize this unique class of phosphoproteins.

Also as described herein, Pin1 is a sequence-specific and phosphorylation-dependent PPIase that can specifically recognize the phosphorylated Ser/Thr-Pro 30 bonds present in mitotic phosphoproteins. These results suggest that Pin1 acts as a general modulator of mitotic phosphoprotein activity, presumably by catalyzing phosphorylation-dependent Pro isomerization.

The crystal structure of human Pin1 complexed with an Ala-Pro dipeptide suggests that the isomerization mechanism of Pin1 includes general acid-base and 35 covalent catalysis during peptide bond isomerization (Ranganathan *et al.*, *Cell*

89:875-886 (1997)). More interestingly, Pin1 displays a unique substrate specificity. It prefers an acidic residue N-terminal to the isomerized Pro bond due to interaction of the acidic side chain with a basic cluster in Pin1. This basic cluster consists of the highly conserved residues Lys63, Arg68, and Arg69 at the entrance to the active site.

- 5 In the crystal structure, this conserved triad sequestered a sulfate ion in close proximity to the β methyl group of the Ala residue in the bound Ala-Pro dipeptide. One candidate for this negatively charged residue is Phospho-Ser/Thr.

To investigate how Pin1 interacts with essential mitotic proteins, a glutathione S-transferase (GST) Pin1 fusion protein was used to screen oriented 10 degenerate peptide libraries. The oriented peptide library approach (Z. Songyang et al. *Cell* 72:767 (1993) was used to screen for optimal peptides. All amino acids except Cys were incorporated at equimolar amounts in each degenerate position, yielding a total theoretical degeneracy for both libraries of $1^9 = 4.7 \times 10^7$ distinct peptide sequences. Pin1-GST beads and MPM2 antibody bound to protein-G 15 beads were incubated with the peptide library mixtures and washed extensively. Bound peptides were eluted with 30% acetic acid and sequenced. The crystal structure of Pin1 containing an Ala-Pro dipeptide substrate revealed a sulfate ion located 5 Å from the C_β carbon of Ala (A), suggesting that phosphorylated Ser (pSer) might be preferred at this site (R. Ranganathan, K. P. Lu, T. Hunter, J. P. 20 Noel, *Cell* 89:875 (1997)).

Next a pS-containing degenerate peptide library of general sequence NH_2 -MAXXXpSXXXAKK, where X includes every amino acid except Cys, was prepared. GST-Pin1 protein preferentially bound a subset of peptides with Pro (P) immediately COOH-terminal to pSer.

25 To investigate whether peptides containing pS-P were preferred substrates for the isomerase activity of Pin1, oligopeptide substrates were synthesized and assayed for *cis/trans* isomerization by Pin1 and by members of the cyclophilin (Cyp18) and FKBP (FHBP12) families of PPIases. The chromogenic oligopeptides were synthesized (A. Bernhardt, M. Drewello, & M. Schutkowski, *Int. J. Peptide* 30 *Protein Res.* 50:143 (1997) and confirmed by NMR. Standard peptides were purchased from Bachem. PPIase activity was assayed and the bimolecular rate constants k_{cat}/K_m were calculated according to the equation $k_{cat}/K_m = (k_{obs} - k_u)/[PPIase]$, where k_u is the first-order rate constant for spontaneous *cis/trans* isomerization and k_{obs} is the pseudo-first-order rate constant for *cis/trans* 35 isomerization in the presence of PPIase, as described in G. Fischer, H. Bang, C.

Mech, *Biomed. Biochim. Acta* 43:1101 (1984) and in J. L. Kofron et al. *Biochemistry* 30:6127 (1991). Affinity of Pin1 for peptides was measured as described in Schutkowski, M., Wöllner, S., & Fischer, G. *Biochemistry* 34:13016, (1995)). Neither Cyp18 nor FKBP12 effectively catalyzed isomerization of peptides with 5 *pS/pT-P* moieties (Table 1). In contrast, either *Y-P* or *pY-P* bonds were good substrates for both enzymes. Thus phosphorylation on *S/T-P*, but not *Y-P*, renders the prolyl-peptidyl bond resistant to the catalytic action of conventional PPIases, and suggests the need for a different enzyme to catalyze this reaction.

Table 1. Interaction between Pin1 and Selected Mitotic Phosphoproteins

	Phosphoproteins	Interphase	Mitotic
10	Cdc25*	-	+++
	Plk1*	-	+++
	Plx1	-	+++
	Wee1	+	++
15	Mos	+	++
	Cdc27*	-	++*
	NIMA	-	+++
	Sox3	-	+++
	Xbr-1b	-	+++
20	MP75 (E-MAP-115)	-	+++
	MP110(Cdc5)	-	+++
	MP68	-	+++
	MP30	-	++
	MP105	+	+
25	MP48	-	-
	Cyclin B*	-	-

The binding between Pin1 and all selected mitotic phosphoproteins was assayed by incubating synthesized proteins with interphase and mitotic *Xenopus* extracts, followed by precipitation with GST-Pin1 beads. The Pin1 interactions with those proteins indicated with * were also confirmed by GST-Pin1 30 pull-down assay from endogenous interphase and mitotic HeLa cell extracts. +, a week but above background interaction; ++, readily detectable interaction; +++, strong interaction.

In contrast to cyclophilins and FKBP_s, Pin1 isomerase activity was highly specific for peptides with pS/pT-P bonds (Table 2). Pin1 displayed little isomerase activity for substrates containing S/T-P bonds. However, phosphorylation of these peptides on S or T residues increased the k_{cat}/K_m values up to 300-fold.

5 Table 2. Sequence-specific and phosphorylation-dependent PPIase activity of Pin1

	Substrate	PPIase Activity kcat/KM (mM ⁻¹ s ⁻¹)
10	AAPL-pNA	55
	AAAPR-pNA	121
	AAPM-pNA	134
	ADPY-pNA	220
	AEPF-pNA	3410
15	AYPY-pNA	5
	<i>A</i> pYpY-pNA	3
	ASPY-pNA	269
	<i>A</i> pSPY-pNA	3370
	ATPY-pNA	635
20	<i>A</i> pTPY-pNA	2480
	AAEPF-pNA	220
	AASPF-pNA	9
	<i>A</i> apSPF-pNA	3760
	AATPF-pNA	4
25	<i>A</i> apTPF-pNA	1370
	AASPR-pNA	7
	<i>A</i> ApSPR-pNA	9300
	WypSPRT-pNA	14100
	<i>A</i> apTPR-pNA	3700

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WFYSPR-pNA	170
WFYpSPR-pNA	20160

Assays were done as described in Table I except that trypsin was used instead of chymotrypsin as an isomer-specific protease when peptides with Pro-Arg-pNA were used as substrates.

Pin1 had low isomerization activity for peptides containing an Ala-Pro peptide bond, whereas incorporation of Glu (E) or Asp (D) immediately preceding P to mimic the *p*S, increased isomerization activity. Peptides containing a Y or *p*Y preceding Pro were poor substrates for Pin1. This substrate specificity distinguishes Pin1 from the conventional PPIases in the cyclophilin and FKBP families.

To further define the sequence specificity of Pin1, a degenerate peptide library containing a fixed *p*S-P sequence flanked by 3 degenerate positions on each side was used. Pin1 selected Arg or aromatic residues at the -1 and +1 positions of the *p*S-P motif (Table 3). Aromatic amino acids were also selected at the -3, Phe/Ile at the -2, and Leu/Ile at the +2 position of *p*S-P. On this basis, several additional peptides were synthesized as Pin1 substrates, as described above. Peptides with Arg introduced at the P+1 position proved better substrates with specificity constants increased up to 1300 fold compared to their non-phosphorylated counterparts (Table 2). Placing aromatic residues NH₂-terminal to the *p*S-P position made these peptides even better substrates (Table 2). The best substrate identified thus far (Trp-Phe-Tyr-*p*Ser-Pro-Arg-pNA) is the optimal sequence selected from the peptide library (Table 2 and 3). The apparent Km of Pin1 towards this peptide was 10 μ M.

Table 3. Binding specificity of Pin1 and MPM-2

	-3	-2	-1	0	+1	+2	+3
Pin1	W	F	Y	pS P	R	L	X
	Y	I	R		F	I	
	F		F		Y		
MPM-2	Y	W	F	pS P	L	X	X
	F	F	L		I		
	W		I		V		

GST-Pin1 and MPM-2 were incubated with the *p*S-P oriented degenerate peptide library NH₂-

- 5 MAXXX*p*SXXXAKK, where X contains every amino acid except Cys. After an extensive wash, peptides bound with GST-Pin1 were eluted and sequenced. Amino acids with a significant selection at each degenerate position are shown.

- As described herein, Pin1 binds a large subset of mitotic phosphoproteins also recognized by the monoclonal antibody MPM-2. Therefore, the sequence 10 specificity for MPM-2 recognition was evaluated. When immobilized MPM-2 antibody was probed with a peptide library containing only a *p*S as the orienting residue, there was a strong selection for peptides with P at the *p*S+1 position. Using the *p*S-P degenerate peptide library, MPM-2 strongly selected peptides with aromatic and aliphatic amino acids at the -3, -1 and +1 positions relative to *p*S-P (Table 3). 15 This MPM-2 binding motif is similar to the sequence motif selected by Pin1 (Table 3) and explains the observation that Pin1 specifically interacts with MPM-2 antigens.

To determine the structural basis for the Pin1 substrate specificity, molecular model-building was performed and tested by site-directed mutagenesis. A peptide 20 (Trp-Phe-Tyr-*p*Ser-Pro-Arg) was modeled into the Pin1 structure (R. Ranganathan, K. P. Lu, T. Hunter, J. P. Noel, *Cell* 89:875 (1997) assuming that the phosphate group of *p*S occupies the position of sulfate in the structure. The phosphate of *p*S in the modeled peptide was superimposed on the co-crystallizing SO₄ ion in the original Pin1 structure, and P residue displacements minimized with respect to the

Ala-Pro ligand in the original Pin1 structure using molecular modeling programs GRASP (A. Nicholls, K. Sharp, B. Honig. *Proteins* 11:281 (1991), Molscript and Raster3d. In this model, R68 and R69 of Pin1 coordinate the *pS* phosphate, a hydrophobic groove accepts the preceding aromatic tripeptide, and the side chain of 5 C113 and H59 coordinate the isomerizing *pS*-P peptide bond with $\omega=90^\circ$, stabilizing the transition state between *cis* and *trans* configuration. To test these predictions from the model, site-directed Pin1 mutants were generated and their PPIase activity assayed as described herein and in K. P. Lu, S. D. Hanes, T. Hunter, *Nature* 380:544 (1996); K. P. Lu and T. Hunter, *Cell* 81:413 (1995).

10 Substitution of both R68 and R69 by Ala reduced the k_{cat}/K_m to 1/ 500 that of wild type Pin1 for the phosphorylated substrate. The catalytic activity of Pin1^{R68,69A} was the same as wild-type Pin1 for the unphosphorylated peptide substrate. Thus, this cluster of basic residues appears to participate in coordinating the phosphate of *pS/pT*. Parvulin (J. U. Rahfeld, et al., *FEBS Lett.* 352:180 (1994); *idid* 343:65 (1994); K. E. Rudd, et al., *TIBS* 20:12 (1995), the prototype of the Pin1 family of 15 PPIases has R68 and R69 replaced by Glu and failed to catalyze the isomerization of *pS*-P peptidyl bonds, though it was very effective in catalyzing the P isomerization of the unphosphorylated peptide. Replacement of the catalytic H59 residue of Pin1 with Ala dramatically decreased the PPIase activity for both phosphorylated and 20 unphosphorylated peptides; however, the specificity for phosphorylated over unphosphorylated substrates was unchanged. For Pin1 the K_{cat}/K_m for the phosphorylated versus unphosphorylated substrate is 19,400/7, which is approximately equal to the similar ratio of 1120/1 for Pin1^{H59A}. Thus H59 appears to play an important role in catalyzing P isomerization and/or binding the substrate P 25 residue.

On the basis of amino acid preferences deduced in the 6 positions surrounding the *pS*-P motif for optimal Pin1 binding, a weighted screening of the SWISS-PROT sequence database. Protein sequence database screening was performed with the program INDOVINATOR, using an entropy-based weighing 30 technique to score for relative information content at each amino acid position

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flanking the pS/pT-P motif with the quantitative peptide library results, which are shown qualitatively in Table 3. This scan revealed within the top 5% of highest scores several potentially important mitotic phosphoprotein targets for Pin1. Many of these proteins are involved in regulation of the cell cycle, cytoskeletal/spindle structure, DNA replication, transcription or RNA processing (Table 4).

TABLE 4

<u>Cell Cycle Regulatory Proteins</u>	<u>Predicted Binding Site(s)</u>	<u>Binding Confirmed</u>
NIMA	YVGTPFYM	Yes
	FYMSPEIC	
	ILNTPVIR	
	ESRTPFTR	
	KSRSPHRR	
	EMPSPFLA	
Cdc25C	YLGSPITT	Yes
10 Plk1	ANITPREG	Yes
Wee1	GRRSPRPD	Yes
Cdc27	FLWSPFES	Yes
Cytoskeletal/Spindle Proteins		
E-MAP-115	ASCSPIM	Yes
15 Centromere protein A	LRKSPFCR	
Nedd 5	YFISPGH	
Nuclear/Splicing/Transcriptional Proteins		
20 Splicing factor SC35	RSRSPRR	Yes
DNA topoisomerase II - alpha	DSASPRYI	
Lim 1 homeobox Protein	FFRSPRRM	

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Laminin beta-1	DPYSPRIQ	
Nuclear pore complex Unp214	FGFSPSGT	
Guanine-Nucleotide-related Proteins		
5 Rab 4	QLRSPRRT	Yes
Rab GDP dissociation inhibitor	YGKSPYLY	
Protein Kinases/Phosphatases		
10 S6 kinase	KIRSPRRF	Yes
Mkk2	PCYTPYYV	
Abl2	GFTTPRLI	
Erk3	WYRSPRLL	
Jnk1,2	FMMTPYVV	
PP2A	WGISPRGA	
15 Tyrosine phosphatase PTP-H1	NWRSPRLR	

Predicted and/or Confirmed Pin1 Substrates

Based on the amino acid preference values in each of the 6 positions surrounding the pS/pT-P motif for optimal Pin1 binding (Table 3), a weighted screening of the SWISS-PROT sequence database was 20 undertaken. This is a partial list of selected proteins with the top scores; human sequences are used whenever possible. Interactions between Pin1 and some of the identified proteins have been confirmed in vitro. Pin1 not only binds these two proteins, but also suppresses their functions ¶, the interaction between Pin1 and SC35 is inferred from their colocalization.

Several of these predicted proteins, such as Rab4, Cdc25 and NIMA, undergo 25 mitosis-specific phosphorylation (R. Heald and F. McKeon, *Cell* 61:579 (1990); E. Bailly, et al., *Nature* 350:715 (1991); A. Blangy, et al., *Cell* 83:1159 (1995); J. Kuang, et al., *Mol. Biol. Cell* 5:135 (1994); X. S. Ye, et al., *EMBO J.* 14:986 (1995)). Cdc25 and NIMA also binds to Pin1 in a phosphorylation-dependent

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manner, as described herein. Other proteins identified in this search, however, had not been previously suspected of interacting with Pin1; therefore, a few were further investigated as example cases. Rab4 and ribosomal S6 kinases were found to interact with Pin1 specifically in mitotic, but not in interphase extracts. Thus, Pin1 binds a 5 wide functional range of mitotic phosphoproteins.

Differences in isomerase activity of Pin1 and other PPIases result from different organization of the X-P binding pocket. In all PPIases, a hydrophobic pocket sequesters the aliphatic P side chain (S. L. Schreiber. *Science* 251:2881 (1991); G. Fischer, *Angew. Chem. Int'l. Ed. Engl.* 33:1415 (1994); F. X. Schmid. *Curr. Biol.* 10 5:993 (1995)), hence the residues responsible for determining substrate preference must reside at the entrance to the P-binding pocket. In Pin1 and its homologues (K. P. Lu, S. D. Hanes, T. Hunter. *Nature* 380:544 (1996); K. P. Lu and T. Hunter, *Cell* 81:413 (1995), S. D. Hanes, et al., *Yeast* 5:55 (1989); R. Maleszka, et al., *Proc. Natl. Acad. Sci. USA* 93:447 (1996)), a cluster of basic residues coordinate the pS 15 phosphate, and determine the specificity of this isomerase. Absence of a basic pocket in the cyclophilins, FKBP, and other members of the parvulin families of PPIases may explain their failure to isomerize the pS/pT-P bonds.

The specificity of Pin1 rationalizes Pin1-binding proteins and also predicts a number of novel potential Pin1 substrates, some of which have been confirmed as in 20 vitro Pin1 targets. Furthermore, Pin1 and MPM-2 bind similar sequences and proteins, and have similar phenotypes, indicating that the wide conservation of MPM-2 epitopes across various species (J. Kuang, et al., *Mol. Biol. Cell* 5:135 (1994); X. S. Ye, et al., *EMBO J.* 14:986 (1995), F. M. Davis, et al., *Proc. Natl. Acad. Sci. USA* 80:2926 (1983); J. M. Westendorf, P. N. Rao, L. Gerace, *idid* 25 91:714-8 (1994); S. Taagepera, et al., *Mol. Biol. Cell* 5:1243 (1994); A. Kumagai, W. G. Dunphy, *Science* 273:1377-80 (1996) can be explained by recognition of this epitope by a highly conserved mitotic regulator, Pin1.

Based on the determination of specific substrates for Pin1, as described herein, 30 inhibitors of Pin1, Pin1-like isomerases and other phospho-Ser/Thr-specific PPIases can be produced. Thus, the present invention provides compounds that inhibit

phosphoserine- and phosphothreonine-specific peptidyl-prolyl isomerasases.

Specifically encompassed by the present invention are peptidyl prolyl isomerasases that recognize phosphorylated serine/threonine-proline bonds present in mitotic phosphoproteins.

5 The inhibitor compounds of the present invention include any molecule that binds into the active site of the phosphoserine- or phosphothreonine-proline specific peptidyl prolyl isomerase and, upon binding to the isomerase, inhibits the isomerase activity. Encompassed by the present invention are inhibitor compounds that mimic the structure and conformation of the substrate moiety when bound to the catalytic

10 site (also referred to herein as the active site) of the isomerase. Molecular inhibitors of the the present invention will typically have an inhibition constant (K_i) of ten micromolar. or less. Specifically encompassed are organic molecules that mimic the structure and conformation of pSer/pThr and bind to the isomerase of interest, thereby inhibiting its activity.

15 The inhibitor compounds of the present invention inculde inhibitors that comprise a core region (or moiety) that mimics the pSer/pThr moiety of the phosphoserine- or phosphothreonine-proline peptidyl prolyl isomerase substrate. Encompassed by the present invention are inhibitors that comprise the pSer/pThr mimic moiety with the mimic moiety being flanked on one side by hydrophobic

20 groups and the other side of the mimic moiety being flanked by hydrophobic or positively charged groups, wherein the groups would contact the active site of the isomerase of interest.

Specifically encompassed by the present invention are inhibitor compounds comprising proteins, polypeptides and peptides. The proteins, polypeptides and 25 peptides of the present invention comprise naturally-occurring amino acids (e.g., L-amino acids), non-naturally amino acids (e.g., D-amino acids), and small molecules that biologically and biochemically mimic the inhibitor peptides, referred to herein as peptide analogs, derivatives or mimetics. (Saragovi, H.U., *et al.*, *Bio/Technology*, 10:773-778 (1992)). The protein, polypeptide or peptide inhibitors of the present 30 invention can be in linear or cyclic conformation.

Compounds that have PPIase inhibiting activity can be identified using oriented degenerate peptide libraries as described herein. For example, a library of xSer/Thr-X-containing peptides of a defined length can be screened for specific binding to the PPIase of interest. Peptides that specifically bind to the PPIase of interest can be 5 further evaluated for PPIase inhibiting activity as described herein.

The phosphoserine and phosphothreonine-specific peptidyl-prolyl isomerase inhibitors, or PPIase inhibitors, of the present invention can comprise a core sequence of xSer/Thr-Y wherein x can be any negatively charged tetra- or pentavalent moiety and Y can be Pro or any Pro analog. Preferred moieties for x can be 10 phosphate, sulfonate, boronate, phosphonate or a sulfonyl amide. The Pro analog can be any nitrogen-containing ring structure, including imidazole, pyrrole, tropolone, benzene, camphor, and heterocyclic aromatic and non-aromatic ring structures. Typically, the xSer/Thr-Y core sequence is flanked by hydrophobic residues or Arg. 15 where the hydrophobic residues (e.g., Phe, Tyr, Trp and Ile) typically precede the xSer/Thr residue and Arg follows the Y residue. Specifically encompassed by the present invention are inhibitors comprising the core sequence phosphoserine/phosphothreonine-proline.

The inhibitors of the present invention can be anywhere from 2 to 200 amino acid residues in length. The inhibitors are typically 2-20 residues in length, and 20 more typically 2-10 residues in length. Most typically the PPIase inhibitors are about eight residues in length, as represented by the consensus sequence, XXXpSer/pThrXXX, wherein X can be any amino acid residue.

Encompassed by the present invention are compounds that are at least eight amino acid residues in length and comprise the core sequence X₁X₂X₃pS-PX₄X₅X₆ 25 wherein each residue can be independently selected as follows X₁ is W, Y or F; X₂ is F or I; X₃ is Y, R, F or W; X₄ is R, F, Y or W; X₅ is L or I and X₆ is any amino acid.

Specifically encompassed by the present invention is the inhibitor of a phosphoserine- or phosphothreonine-proline-specific peptide prolyl isomerase comprising Trp-Phen-Tyr-pSer-Pro-Arg.

The inhibitors of the present invention can be synthesized using standard laboratory methods that are well-known to those of skill in the art, including standard solid phase techniques. Inhibitors comprising naturally occurring amino acids can also be produced by recombinant DNA techniques known to those of skill, 5 and subsequently phosphorylated.

The inhibitors of the present invention can comprise either the 20 naturally occurring amino acids or other synthetic amino acids. Synthetic amino acids encompassed by the present invention include, for example, naphthylalanine, L-10 hydroxypropylglycine, L-3,4-dihydroxyphenylalanyl, α -amino acids such as L- α -hydroxylysyl and D- α -methylalanyl, L- α -methyl-alanyl, β amino-acids such as β -analine, and isoquinolyl.

D-amino acids and other non-naturally occurring synthetic amino acids can also be incorporated into the inhibitors of the present invention. Such other non-naturally occurring synthetic amino acids include those where the naturally occurring side 15 chains of the 20 genetically encoded amino acids (or any L or D amino acid) are replaced with other side chains, for instance with groups such as alkyl, lower alkyl, cyclic 4-, 5-, 6-, to 7-membered alkyl, amide, amide lower alkyl, amide di(lower alkyl), lower alkoxy, hydroxy, carboxy and the lower ester derivatives thereof, and with 4-, 5-, 6-, to 7-membered heterocyclic. In particular, proline analogs in which 20 the ring size of the proline residue is changed from 5 members to 4,6, or 7 members can be employed.

As used herein, "lower alkyl" refers to straight and branched chain alkyl groups having from 1 to 6 carbon atoms, such as methyl, ethyl propyl, butyl and so on. "Lower alkoxy" encompasses straight and branched chain alkoxy groups having 25 from 1 to 6 carbon atoms, such as methoxy, ethoxy and so on.

Cyclic groups can be saturated or unsaturated, and if unsaturated, can be aromatic or non-aromatic. Heterocyclic groups typically contain one or more nitrogen, oxygen, and/or sulphur heteroatoms, e.g., furazanyl, furyl, imidazolidinyl, imidazolyl, imidazolinyl, isothiazolyl, isoxazolyl, morpholinyl (e.g. morpholino), 30 oxazolyl, piperazinyl (e.g. 1-piperazinyl), piperidyl (e.g. 1-piperidyl, piperidino),

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pyranyl, pyrazinyl, pyrazolidinyl, pyrazolinyl, pyrazolyl, pyridazinyl, pyridyl, pyrimidinyl, pyrrolidinyl (e.g. 1-pyrrolidinyl), pyrrolinyl, pyrrolyl, thiadiazolyl, thiazolyl, thienyl, thiomorpholiny (e.g. thiomorpholino), and triazolyl. The heterocyclic groups can be substituted or unsubstituted. Where a group is

- 5 substituted, the substituent can be alkyl, alkoxy, halogen, oxygen, or substituted or unsubstituted phenyl. (U.S. Patent No. 5,654,276 and U.S. Patent No. 5,643,873).

Biologically active derivatives or analogs of the above-described inhibitors, referred to herein as peptide mimetics, can be designed and produced by techniques known to those of skill in the art. (U.S. Patent Nos. 4,612,132; 5,643,873 and 10 5,654,276) These mimetics are based on a specific peptide PPIase inhibitor sequence and maintain the relative positions in space of the corresponding peptide inhibitor. These peptide mimetics possess biologically activity (i.e., PPIase inhibiting activity) similar to the biological activity of the corresponding peptide compound, but possess a "biological advantage" over the corresponding peptide inhibitor with respect to 15 one, or more, of the following properties: solubility, stability, and susceptibility to hydrolysis and proteolysis.

Methods for preparing peptide mimetics include modifying the N-terminal amino group, the C-terminal carboxyl group, and/or changing one or more of the amino linkages in the peptide to a non-amino linkage. Two or more such 20 modifications can be coupled in one peptide mimetic inhibitor. The following are examples of modifications of peptides to produce peptide mimetics as described in U.S Patent Nos: 5,643,873 and 5,654,276; the same techniques are used to prepare mimetics of the PPIase inhibitors described herein.

MODIFICATION OF THE N-AMINO TERMINUS

25 After solid phase synthesis of the peptide inhibitor, the blocking group on the N-terminus amino group can be selectively removed so as to provide for a peptide sequence blocked at all positions other than the N-terminal amino group and attached to a solid resin through the C-terminus. One can then modify the amino

terminus of the peptides of the invention to produce peptide mimetics of the invention.

Amino terminus modifications include alkylating, acetylating, adding a carbobenzoyl group, forming a succinimide group, etc. Specifically, the N-terminal 5 amino group can then be reacted as follows:

- (1) to form an amide group of the formula $RC(O)NH-$ where R is as defined above by reaction with an acid halide (e.g., $RC(O)Cl$) or acid anhydride. Typically, the reaction can be conducted by contacting about equimolar or excess amounts (e.g., about 5 equivalents) of an acid halide to the peptide in an inert diluent 10 (e.g., dichloromethane) preferably containing an excess (e.g., about 10 equivalents) of a tertiary amine, such as diisopropylethylamine, to a scavenge the acid generated during reaction. Reaction conditions are otherwise conventional (e.g., room temperature for 30 minutes). Alkylation of the terminal amino to provide for a lower alkyl N-substitution followed by reaction with an 15 acid halide as described above will provide for N-alkylamide group of the formula $RC(O)NR-$;
- (b) to form a succinimide group by reaction with succinic anhydride. As before, an approximately equimolar amount or an excess of succinic anhydride (e.g., about 5 equivalents) can be employed and the amino group is converted to the 20 succinimide by methods well known in the art including the use of an excess (e.g., ten equivalents) of a tertiary amine such as diisopropylethylamine in a suitable inert solvent (e.g., dichloromethane). See, for example, Wollenberg, et al., U.S. Pat. No. 4,612,132 which is incorporated herein by reference in its entirety. It is understood that the succinic group can be substituted with, for 25 example, C_2-C_6 alkyl or -SR substituents which are prepared in a conventional manner to provide for substituted succinimide at the N-terminus of the peptide. Such alkyl substituents are prepared by reaction of a lower olefin (C_2-C_6) with maleic anhydride in the manner described by Wollenberg, et al., supra. and -SR substituents are prepared by reaction of RSH with maleic anhydride where R is 30 as defined above;

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- (c) to form a benzyloxycarbonyl-NH- or a substituted benzyloxycarbonyl-NH-group by reaction with approximately an equivalent amount or an excess of CBZ-Cl (i.e., benzyloxycarbonyl chloride) or a substituted CBZ-Cl in a suitable inert diluent (e.g., dichloromethane) preferably containing a tertiary amine to 5 scavenge the acid generated during the reaction;
- (d) to form a sulfonamide group by reaction with an equivalent amount or an excess (e.g., 5 equivalents) of R-S(O)₂Cl in a suitable inert diluent (dichloromethane) to convert the terminal amine into a sulfonamide where R is as defined above. Preferably, the inert diluent contains excess tertiary amine (e.g., ten equivalents) 10 such as diisopropylethylamine, to scavenge the acid generated during reaction. Reaction conditions are otherwise conventional (e.g., room temperature for 30 minutes);
- (e) to form a carbamate group by reaction with an equivalent amount or an excess (e.g., 5 equivalents) of R-OC(O)Cl or R-OC(O)OC₆H₄-p-NO₂ in a suitable inert 15 diluent (e.g., dichloromethane) to convert the terminal amine into a carbamate where R is as defined above. Preferably, the inert diluent contains an excess (e.g., about 10 equivalents) of a tertiary amine, such as diisopropylethylamine, to scavenge any acid generated during reaction. Reaction conditions are otherwise conventional (e.g., room temperature for 30 minutes); and
- 20 (f) to form a urea group by reaction with an equivalent amount or an excess (e.g., 5 equivalents) of R-N=C=O in a suitable inert diluent (e.g., dichloromethane) to convert the terminal amine into a urea (i.e., RNHC(O)NH-) group where R is as defined above. Preferably, the inert diluent contains an excess (e.g., about 10 equivalents) of a tertiary amine, such as diisopropylethylamine. Reaction 25 conditions are otherwise conventional (e.g., room temperature for about 30 minutes).

MODIFICATION OF THE C-TERMINUS

In preparing peptide mimetics wherein the C-terminal carboxyl group is replaced by an ester (i.e., -C(O)OR where R is as defined above), the resins used to prepare the peptide acids are employed, and the side chain protected peptide is cleaved with 5 base and the appropriate alcohol, e.g., methanol. Side chain protecting groups are then removed in the usual fashion by treatment with hydrogen fluoride to obtain the desired ester.

In preparing peptide mimetics wherein the C-terminal carboxyl group is replaced by the amide -C(O)NR³R⁴, a benzhydrylamine resin is used as the solid support for 10 peptide synthesis. Upon completion of the synthesis, hydrogen fluoride treatment to release the peptide from the support results directly in the free peptide amide (i.e., the C-terminus is -C(O)NH₂). Alternatively, use of the chloromethylated resin during peptide synthesis coupled with reaction with ammonia to cleave the side chain protected peptide from the support yields the free peptide amide and reaction 15 with an alkylamine or a dialkylamine yields a side chain protected alkylamide or dialkylamide (i.e., the C-terminus is -C(O)NRR¹ where R and R¹ are as defined above). Side chain protection is then removed in the usual fashion by treatment with hydrogen fluoride to give the free amides, alkylamides, or dialkylamides.

Alternatively, the C-terminal carboxyl group or a C-terminal ester can be 20 induced to cyclize by internal displacement of the -OH or the ester (-OR) of the carboxyl group or ester respectively with the N-terminal amino group to form a cyclic peptide. For example, after synthesis and cleavage to give the peptide acid, the free acid is converted to an activated ester by an appropriate carboxyl group activator such as dicyclohexylcarbodiimide (DCC) in solution, for example, in 25 methylene chloride (CH₂Cl₂), dimethyl formamide (DMF) mixtures. The cyclic peptide is then formed by internal displacement of the activated ester with the N-terminal amine. Internal cyclization as opposed to polymerization can be enhanced by use of very dilute solutions. Such methods are well known in the art.

MODIFICATION TO INCORPORATE A NON-PEPTIDYL LINKAGE

Peptide mimetics wherein one or more of the peptidyl linkages [-C(O)NH-] have been replaced by such linkages as a -CH₂-carbamate linkage, a phosphonate linkage, a -CH₂-sulfonamide linkage, a urea linkage, a secondary amine (-C₂NH-) linkage, 5 and an alkylated peptidyl linkage [-C(O)NR⁶- where R⁶ is lower alkyl] are prepared during conventional peptide synthesis by merely substituting a suitably protected amino acid analogue for the amino acid reagent at the appropriate point during synthesis.

Suitable reagents include, for example, amino acid analogs wherein the carboxyl 10 group of the amino acid has been replaced with a moiety suitable for forming one of the above linkages. For example, if one desires to replace a -C(O)NR- linkage in the peptide with a -CH₂- carbamate linkage (-CH₂OC(O)NR-), then the carboxyl (-COOH) group of a suitably protected amino acid is first reduced to the -CH₂OH group which then converted by conventional methods to a -OC(O)Cl functionality or 15 a para-nitrocarbonate -OC(O)O-C₆H₄-p-NO₂ functionality. Reaction of either of such functional groups with the free amine or an alkylated amine on the N-terminus of the partially fabricated peptide found on the solid support leads to the formation of a -CH₂OC(O)NR-linkage. For a more detailed description of the formation of such -CH₂-carbamate linkages.

20 Similarly, replacement of an amino linkage in the peptide with a phosphonate linkage can be achieved using techniques known to those of skill in the art.

Replacement of an amino linkage in the peptide with a -CH₂-sulfonamide linkage can be achieved by reducing the carboxyl (-COOH) group of a suitably 25 protected amino acid to the -CH₂OH group and the hydroxyl group is then converted to a suitable leaving group such as a tosyl group by conventional methods. Reaction of the tosylated derivative with, for example, thioacetic acid followed by hydrolysis and oxidative chlorination will provide for the -CH₂-S(O)₂Cl functional group which replaces the carboxyl group of the otherwise suitably protected amino acid. Use of this suitably protected amino acid analogue in peptide synthesis provides for

inclusion of an -CH₂S(O)₂NR- linkage which replaces the amino linkage in the peptide thereby providing a peptide mimetic.

Replacement of an amino linkage in the peptide with a urea linkage can be achieved using techniques known to those of skill in the art.

- 5 Secondary amine linkages wherein a -CH₂NH- linkage replaces the amino linkage in the peptide can be prepared by employing, for example, a suitably protected dipeptide analogue wherein the carbonyl bond of the amino linkage has been reduced to a CH₂ group by conventional methods. For example, in the case of diglycine, reduction of the amide to the amine will yield after deprotection
- 10 H₂NCH₂CH₂NHCH₂COOH which is then used in N-protected form in the next coupling reaction. The preparation of such analogues by reduction of the carbonyl group of the amino linkage in the dipeptide is well known in the art.

The suitably protected amino acid analog is employed in the conventional peptide synthesis in the same manner as would the corresponding amino acid. For 15 example, typically about 3 equivalents of the protected amino acid analogue are employed in this reaction. An inert organic diluent such as methylene chloride or DMF is employed and, when an acid is generated as a reaction by-product, the reaction solvent will typically contain an excess amount of a tertiary amine to scavenge the acid generated during the reaction. One particularly preferred tertiary 20 amine is disopropylethylamine which is typically employed in about 10 fold excess. The reaction results in incorporation into the peptide mimetic of an amino acid analogue having a non-peptidyl linkage. Such substitution can be repeated as desired such that from zero to all of the amino bonds in the peptide have been replaced by non-amino bonds.

25 The inhibitors of the present invention can also be cyclic protein, peptides and cyclic peptide mimetics. Such cyclic inhibitors can be produced using known laboratory techniques, e.g., as described in U.S.S.N. 08/864,392, filed on May 28, 1997 and U.S Patent No:5,654,276.

Inhibitors of the present invention are evaluated for biological activity as 30 described herein. For example, the candidate compounds can be screened in an

assay that determines the displacement of a labeled high affinity molecule (e.g., a competitive inhibition assay) in an assay utilizing immobilized molecules on a grid, as well as screening libraries of candidate molecules. These techniques are known to those of skill in the art.

5 As defined herein, biological activity of the PPIase inhibitors include specific binding to the PPIase of interest (e.g., specific binding to Pin1) and/or specific inhibition of the peptidyl prolyl isomerase activity as measured as described in Schutkowski, M. *et al.*, *Biochemistry*, 34:13016 (1995). Specific binding to the PPIase of interest can be determined as described herein. Further evaluation of 10 candidate inhibitors (e.g., inhibitors that specifically bind to the PPIase of interest, for inhibiting activity can be determined by competitive inhibition assay. Alternatively, candidate molecules of the present invention can be directly evaluated for their inhibitory activity without prior determination of their specific binding to the isomerase of interest. Inhibitor compounds of the present invention 15 typically have a K_i in the nanomolar or micromolar range. Methods to determine K_i are known to those of skill in the art.

The inhibitors of the present invention can be used *in vitro* to study cell cycle regulation and mitotic events. For example, the inhibitors of the present invention can be used to evaluate mitotic events in mammalian cells by inhibiting a specific 20 isomerase and evaluating the effects on the cell cycle.

The inhibitors of the present invention can also be used to interfere with eucaryotic cell growth. The inhibitors can be used to inhibit cell growth, and to kill targeted cells. For example, the inhibitors of the present invention can be used to treat fungal and yeast, including Aspergillus, and parasitic infections (e.g., malaria) 25 in mammals. As defined herein, mammals include domesticated animals and humans. Specifically, the inhibitors of the present invention can be used to treat hyperplastic and neoplastic disorders in mammals, including humans.

For example, Pin1 is an important molecule in controlling the sequential events of mitosis (Figure 1). Entry and exit from mitosis are accompanied by abrupt 30 changes in kinase activities, which lead to changes in the phosphorylation state of

numerous proteins that trigger specific events in mitosis. Pin1 binding and consequent inhibition of target protein activity may provide a means for temporally synchronizing and/or amplifying the activity of mitotic proteins. Inhibition of Pin1 induces mitotic arrest and apoptosis. Thus, the Pin1 mediated mechanism of 5 regulating mitotic events is a therapeutic target for cancer.

Neoplastic and hyperplastic disorders include all forms of malignancies, psoriasis, retinosis, athrosclerosis resulting from plaque formation, leukemias and benign tumor growth. For example, such disorders include lymphomas, papilomas, pulmonary fibrosis, rheumatoid arthritis and multiple sclerosis.

10 The inhibitors of the present invention can be formulated into compositions with an effective amount of the inhibitor as the active ingredient. Such compositions can also comprise a pharmaceutically acceptable carrier, and are referred to herein as pharmaceutical compositions. The inhibitor compositions of the present invention can be administered intraveneously, parenterally, orally, by inhalation or by 15 suppository. The inhibitor composition may be administered in a single dose or in more than one dose over a period of time to achieve a level of inhibitor which is sufficient to confer the desired effect.

Suitable pharmaceutical carriers include, but are not limited to water, salt 20 solutions, alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrrolidone, etc. The pharmaceutical preparations can be sterilized and desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, and/or aromatic substances and the like which 25 do not deleteriously react with the active compounds. They can also be combined where desired with other active agents, e.g., enzyme inhibitors, to reduce metabolic degradation.

For parenteral application, particularly suitable are injectable, sterile solutions, 30 preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. Ampoules are convenient unit dosages.

It will be appreciated that the actual effective amounts of the inhibitor in a specific case will vary according to the specific compound being utilized, the particular composition formulated, the mode of administration and the age, weight and condition of the patient, for example. As used herein, an effective amount of 5 inhibitor is an amount of inhibitor which is capable of inhibiting the isomerase activity of the isomerase of interest, thereby inhibiting target cell growth and resulting in target cell death. Dosages for a particular patient can be determined by one of ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol).

10 The present invention is illustrated by the following examples, which are not intended to be limited in any way.

Example 1: Expression, Purification and Kinetic Analysis of Recombinant Pin1 Proteins

Pin1 was expressed and purified by Ni^{2+} -NTA agarose column as an N-15 terminally His₆-tagged fusion protein, followed by removing the tag using thrombin, as described in Lu et al., 1996; and Ranganathan et al., 1997). To generate an N-terminally GST-Pin1 fusion protein, Pin1 cDNA was subcloned into a pGEX vector and the resulting fusion protein was expressed and purified by glutathione agarose column, as described in Lu et al., 1993; Lu, et al., 1996. GST-Pin1 was stored in the 20 agarose bead at 4 °C for 2 weeks or eluted from the beads and concentrated to 20 mg/ml with a Centricon-10 (Amicon), followed by storing at -80 °C. Both preparations were stored in a buffer containing 20 mM HEPES, pH 7.5, 50 mM NaCl and 1 mM DTT, as described in Ranganathan et al., 1997. All proteins were quantified by the method of Bradford (Biorad) using BSA as a standard.

25 Site-directed mutations of Pin1 were introduced using PCR-based techniques and verified by DNA sequencing. The corresponding mutant proteins were expressed and purified using the same procedures as those described for wild-type Pin1. PPIase activity was measured, as described previously (Lu et al., 1996), with the exception that the absorbance of *p*-nitroaniline (at 395 nM) was followed every

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second for 2-10 min. and data were analyzed offline using a kinetic computer program written by G. Tucker-Kellogg in the C. Walsh lab at Harvard Medical School.

Example 2: Analysis of Pin1 and Its Binding Proteins During Cell Cycle

5 HeLa cells were arrested at the G1/S boundary using double thymidine and aphidicolin block, and released to enter the cell cycle, as described in Heintz, N., H.L. Sive and R.G. Roeder, *Mol Cell Biol* 3:539-550 (1983) and Lu, K.P. and T. Hunter, *Cell* 81:413-424 (1995)).
To accumulate cells at mitosis, nocodazole (50 ng/ml) was added to cells at 8 h after
10 the release for the specified period of time. To obtain a large quantity of interphase and mitotic cells, HeLa cells were incubated with double thymidine and aphidicolin or nocodazole for 16 h, which resulted in over 90% of cells being arrested at the G1/S boundary or mitosis, respectively. Cells were harvested and a aliquot of cells was subjected to flow cytometry analysis, as described in Lu and Hunter, 1995). The
15 remaining cells were lysed in RIPA buffer (10 mM sodium phosphate pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM sodium orthovanadate, 10 µg/ml aprotinin, 50 µg/ml phenylmethylsulfonyl fluoride and 1 mM DTT) and same amount of total proteins were subjected to immunoblotting analysis using various antibodies or Farwestern analysis using GST-
20 Pin1 as a probe. For Farwestern analysis, after blocking with 5% BSA, membranes were incubated with 2 µg/ml GST-Pin1 in TBST for 2 hr, followed by incubation with anti-GST monoclonal antibodies (UBI) and the ECL detection procedures.

Example 3: Microinjection of *Xenopus* Embryos

Unfertilized eggs were incubated with sperm, dejellied, and 4 µM of the
25 indicated protein (about 10 fold above the estimated endogenous levels) was injected in one cell of two cell stage embryos (30 embryos each protein). The injected embryos were allowed to develop at 18°C to stage 8 and pictures were taken of typical embryos. The titration of Pin1 and the mutants was essentially as described

above except that the indicated protein was injected into one cell of 4 cell stage (18 embryos each Pin1 concentration) to the indicated final concentration and allowed to develop for 3h. The cell cycle blocks by GST-Pin1 were not homogeneous as cells that were injected with greater concentrations of GST-Pin1 were cleaved fewer times 5 indicating a tighter cell cycle block. To be consistent, cell cycle blocked embryo's were scored as those that contained at least one cell on the injected side that was greater than 5 times larger than uninjected cells (Figure 2).

Example 4: Preparation of *Xenopus* CSF Extracts

Xenopus CSF extracts were prepared from unfertilized eggs, as previously 10 described (Murray, 1991) and used immediately. To examine the effect of Pin1 on mitotic entry, a fresh CSF extract containing demembranated sperm (150/ μ l) and rhodamine tubulin (2 μ g/ml) was activated by addition of 0.4 mM calcium chloride for 15 min, before the indicated concentrations of various Pin1 proteins were added and mitotic entry was followed for 2 h by nuclear morphology, nuclear envelope 15 breakdown, spindle formation and Cdc2 activity, as described previously (Murray, A.W., *Methods Cell Biol* 36:581-605 (1991)). The cell cycle state of nuclei within the extracts were over 90% synchronous and typical nuclei were photographed.

Example 5: Synthesis of Mitotic Phosphoproteins

The mitotic phosphoproteins were translated *in vitro* using the TNT coupled 20 transcription/translation kit (Promega) in a total volume of 10 μ l in the presence of 8 μ Ci [35 S]methionine (1000 Ci/mmol) for 2 h at 30°C. They were then incubated in *Xenopus* interphase and mitotic extracts as described (Stukenberg et al., 1997). These incubated clones were precipitated by Pin1 beads as described below. The 25 *Xenopus* Mos and Wee1 clones were a kind gift of M. Murakami, G. F. Woude and J. Cooper; the *Xenopus* Cdc25 clone was a generous gift of W. Dunphy, T3 and T3S2 Cdc25 mutants were kindly provided by J. Maller (Izumi and Maller, 1993).

Example 6: Production of Pin1 and Cdc25 Antibodies.

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Since antibodies that we previously raised against C-terminal peptide of Pin1 (Lu, et al., 1996) did not have a high sensitivity for detecting Pin1, especially for Xenopus Pin1, rabbits were immunized with His-Pin1 as an antigen. After 2 months, antisera specifically recognize a single 18 kDa Pin1 protein in human cells and Xenopus extracts.

To raise antibodies against Xenopus Cdc25, recombinant GST-Cdc25 (the clone was a kind gift of A. Nebraska and T. Hunt) was affinity purified as described by the manufacture (Pharmacia). The protein was further purified by SDS-PAGE and a gel slice containing Cdc25 was used to immunize rabbits.

10 Example 7: GST Pull-down, Immunoprecipitation and Immunoblotting Analysis

To detect Pin1-binding proteins, either HeLa cells were lysed in or *Xenopus* extracts were diluted in a buffer (buffer A) containing 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate, 10% glycerol, 1% Triton X100, 10 µg/ml aprotinin, 50 µg/ml phenylmethylsulfonyl fluoride and 1 mM DTT. 15 The lysates were preclarified with boiled *S. aureus* bacteria (CalBiochem) and then incubated with 10 µl of agarose beads containing various GST-Pin1 proteins or control GST for 2 h at 4°C. The precipitated proteins were washed 5 times in the same buffer and subjected to immunoblotting analysis. Immunoprecipitation and immunoblotting analysis using MPM-2 antibody (Davis et al. 1983), which was 20 kindly provided by J. Kuang, Pin1 antibodies (Lu et al., 1996, kindly provided by M. White or newly generated), anti-phospho.Tyr antibody (UBI), anti-Cdc25C (Ogg, et al., 1994) (from H. Piwnica-Worms and Santa Cruz Biotechnology), anti-Cdc27, anti-Plk1 (Zymed), anti-Cdc2 (Solomon, M.J., M. Glotzer, T.H. Lee, M. Philippe and M.W. Kirschner. *Cell* 63:1013-1024 (1991), anti-human Myt1, anti-human 25 cyclin B1 and anti-*Xenopus* cyclin B were performed, as described previously in Lu and Hunter, 1995; Lu et al., 1996).

Example 8: Coimmunoprecipitation of Pin1 and Cdc25

To detect Pin1 and Cdc25 interaction during the *Xenopus* cell cycle, about 500 eggs were fertilized in a minimal volume of MMR (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 2mM CaCl₂, 0.1 mM EDTA, 5 mM HEPES, pH 7.8), diluted in 0.1X MMR for 10 minutes, dejellied as described in Murray, (1991) and incubated in CSF-XB 5 (100 mM KCl, 0.1 mM CaCl₂, 2 mM MgCl₂, 10 mM K-HEPES, pH 7.7, 50 mM Sucrose 5 mM EGTA, pH 7.7). At the indicated time after fertilization 15 eggs were crushed into 150 μ l of ice cold CSF-XB with 1 μ M okadaic Acid, microcentrifuged for 20 seconds, the layer between the yolk and the pellet was removed to a fresh chilled tube. This solution was mixed well and 5 μ l was frozen in liquid nitrogen for 10 future H1 kinase assays, and 30 μ l was diluted in 10 μ l of either α -cdc25 or control rabbit sera beads in 100 μ l of buffer A (containing 5 mM EDTA and 1 μ M microcystein but not vanadate). The immunoprecipitation reactions were rotated for 15 approximately 40 minutes at 4°C, washed 4 times in and subjected to immunoblotting with anti-Pin1 antibodies. The associated Pin1 was quantified as described (Stukenberg et al., 1997).

Example 9: Cdc2 and Cdc25 Assays

Cdc2 was assayed using histone H1 as a substrate, as previously described in Murray, 1991; Lu and Hunter, (1995). Cdc25 activity was assayed by using the activation of its endogenous substrate, Cdc2/cyclin B complex phosphorylated on 20 Thr161, Tyr15, Thr14 as an indicator using a variation of an established protocol (Kumagai, A. and W.G. Dunphy. 1996. *Science* 273:1377-1380 (1996). When cyclin B is added to a *Xenopus* interphase extract at levels insufficient to activate mitosis (referred to as a "subthreshold cyclin concentration"), the added cyclin B binds Cdc2 and the Cdc2 in the complex is phosphorylated by CAK, Wee1 and Myt 25 1 to accumulate in an inactive form (Solomon et al. 1991).

A subthreshold concentration of GST cyclin B (10 μ g) was added to 1 ml of *Xenopus* interphase extract for thirty min at room temperature (Solomon, M.J., M. Glotzer, T.H. Lee, M. Philippe and M.W. Kirschner, *Cell* 63:1013-1024 (1990). This was diluted 8 fold in XB+ 3 mM DTT, rotated for 1 h with 3 ml of GST

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agarose beads, washed 3 times in XBIP (XB+ 500 mM NaCl and 1% NP40 + 2 mM DTT), washed 2 times (once overnight) in EB (80 mM β glycerol phosphate, 15 mM EGTA, 15 mM MgCl₂) + 2 mM DTT, 500 mM NaCl and 1% NP40, and finally twice with EB + 10 mM DTT. These Cdc25 assay beads were stored at 4°C for up to 5 1 month.

Mitotic GST-Cdc25 was purified by incubating 22 μ g of GST-Cdc25, in a *Xenopus* mitotic extract for 30 min at 23°C, this was diluted 8 fold in XB and rotated with 50 μ l of glutathione-Agarose beads (Sigma) for 1 hr at 4°C. The beads were washed 5 times in XB-IP, twice in XB+2 mM DTT and eluted in 25 μ l XB + 2 10 mM reduced GSH. The final concentration of Mitotic GST-Cdc25 was 0.36 mg/ml. A 27 fold dilution of this mitotic GST-Cdc25 could fully activate Cdc2 in the assay below, while GST-cdc25 isolated from Interphase extracts in parallel lost activity after a 3 fold dilution. Thus the mitotic extract stimulated the Cdc25 at least 9 times over interphase extracts. To assay Cdc25 activity 1 μ M mitotic GST-cdc25, and the 15 indicated concentration of either Pin1, Pin1^{R68,69A} or BSA were incubated in a 20 μ l reaction in XB + 1 mM ATP for 10 minutes at room temperature. These reactions were sequentially diluted (1/1, 1/3, 1/9, 1/27) into XB + 1mM ATP and 10 μ l of each was mixed with 10 μ l of cdc25 assay beads for 10 minutes at room temperature with constant shaking. The Cdc25 assay beads were washed 3 times in XB-IP, 2 20 times in EB+1mM DTT and assayed for H1 kinase activity. Phosphoimager analysis of the H1 kinase assays were quantified by the Molecular Dynamics ImageQuant 3.3 software. As described herein an assay with 1 μ M mitotic GST-cdc25, 0.67 μ M of either Pin1, Pin1^{R68,69A} or 16 μ M BSA then diluted 27 fold before being mixed with the Cdc25 assay beads and the amount of H1 kinase activity is relative to the amount 25 of activity of the beads without cdc25 being zero and the BSA reaction being 100%. The most reproducible way to quantify the Cdc25 activity in this assay was by determining the endpoint dilution of Cdc25 which could activate Cdc2. Therefore the Cdc25 activity is quantified by the endpoint dilution of the mitotic GST-Cdc25 at which Cdc2 on the beads could still be significantly activated.

Example 10: Pin1 Levels are Constant through the Cell Cycle

Whereas overexpression of Pin1 results in G2 arrest, depletion of Pin1 induces mitotic arrest without affecting DNA synthesis. To determine the basis for this cell cycle-specificity, it was determined whether Pin1 protein level fluctuated during the 5 cell cycle. To address this question, HeLa cells were synchronized at the G1/S boundary. At different times following the release from the block, cells were harvested and analyzed by flow cytometry or lysed and analyzed for protein expression by immunoblotting. Analysis of DNA content and cyclin B1 levels indicated that the HeLa cells synchronously progressed through different phases of 10 the cell cycle. However, total Pin1 levels did not change significantly during the cell cycle.

Example 12: Pin1 Directly Binds a Subset of Conserved Mitotic Phosphoproteins

Since the levels of Pin1 do not fluctuate during the cell cycle, its mitosis-specific function is likely conferred by some other mechanisms. There are many such 15 possibilities. Pin1 could be subjected to post-translational modifications, such as phosphorylation, or allosteric interactions with a transiently appearing subunit, like a cyclin which regulates its activity. Alternatively, the interaction of Pin1 and its targets may be cell cycle-regulated. Initial experiments suggested no evidence for 20 Pin1 phosphorylation or for interaction of Pin1 with a regulatory subunit. A cell cycle-dependent interaction of Pin1 with its binding proteins was tested for.

A glutathione-S-transferase (GST) fusion protein containing full length Pin1 was bacterially expressed, purified, and then used to probe for interacting proteins in S-phase, mitosis or G1-phase by Farwestern analysis. The ability of Pin1 to interact with cellular proteins remained relatively low during S, increased when cells 25 progressed through G2/M (10 h point), and was almost completely lost when cells moved to the next G1 (14 h point). However, if cells were not allowed to progress into the next cell cycle, but rather were blocked at mitosis by adding nocodazole (14+Noc), Pin1-binding activity increased even further. Since the binding activity was detected using denatured proteins, the protein-protein interaction between Pin1

and these proteins must be direct. To examine whether this Pin1 interaction with its target proteins occur under nondenaturing conditions and to estimate the number of Pin1-interacting proteins, glutathione beads containing GST and GST-Pin1 were incubated with interphase and mitotic extracts, and beads were extensively washed 5 and proteins bound to beads were separated on SDS-containing gels and stained with Coomassie blue. Whereas GST did not precipitated any detectable proteins from either interphase or mitotic extracts, GST-Pin1 specifically precipitated about 30 clearly Coomassie-stainable bands from mitotic extracts, but only 4-7 minor bands from interphase extracts. Together, these two results indicate that Pin1 mainly 10 interacts with a subset of proteins in a mitosis-specific manner.

The crystal structure of Pin1 suggests that Pin1 could strongly interact with a Phospho.Ser/Thr-Pro motif (Ranganathan, *et al.*, *Cell* 89:875-886 (1997)). A large number of proteins have been shown to be phosphorylated at such a motif specifically during mitosis and many of these phosphoproteins are recognized by the 15 MPM-2 antibody. Therefore, interactions between Pin1 and MPM-2 antigens were examined. After incubation with soluble proteins prepared from interphase and mitotic HeLa cells, GST-Pin1 and control GST glutathione beads were washed extensively and the interacting proteins are detected by immunoblotting with the MPM-2 antibody. Many of the GST-Pin1-binding proteins reacted with MPM-2 only 20 in the mitotic extracts, including a strong band of 55 kDa (p55). p55 has been previously shown to be the most prominent MPM-2 antigen in HeLa cells (Zhao *et* al., *FEBS Lett* 249:389-395 (1989), although its identity remains to be determined. In contrast, control GST glutathione beads precipitated just two proteins (p58/60) 25 from either lysate. In addition, when MPM-2 immunoprecipitates were subjected to Farwestern analysis using GST-Pin1 as a probe, Pin1 directly bound MPM-2 antigens on membranes.

To determine whether GST-Pin1 can deplete MPM-2 antigens and to estimate what concentrations of Pin1 are required to completely deplete MPM-2 antigens, mitotic extracts were incubated with different amounts of GST-Pin1, followed by 30 analyzing MPM-2 antigens remaining in the depleted supernatants. The total

cellular Pin1 concentration in HeLa cells was estimated to be about 0.5 μ M, based on immunoblotting analysis using anti-Pin1 antibodies with recombinant Pin1 protein as a standard. At a concentration (8 μ M) that was about 15 fold higher than the endogenous level, Pin1 depleted the majority of MPM-2 antigens, indicating that 5 Pin1 strongly interacts with most MPM-2 antigens. The above results demonstrate that Pin1 interacts with MPM-2 antigens in vitro.

To determine if endogenous Pin1 interacts with MPM-2 antigens in the cell, Pin1 was immunoprecipitated from either interphase or mitotic HeLa extracts using anti-Pin1 antibodies in the presence of various phosphatase inhibitors. The resulting 10 Pin1 immunoprecipitates were probed with MPM-2. As described above, several MPM-2 antigens were co-immunoprecipitated with anti-Pin1 antibodies. These results indicate that stable complexes between Pin1 and MPM-2 antigens exist in the cell and that Pin1 does not form complexes with all Pin1-binding proteins at the same time *in vivo*.

15 Since Pin1 and MPM-2 antigens are highly conserved, it is possible that Pin1-binding proteins are also conserved. To examine this possibility, the interaction between human Pin1 and mitotic phosphoproteins in *Xenopus* extracts was observed. When GST-Pin1 was incubated with interphase or mitotic egg extracts, Pin1 specifically precipitated a subset of MPM-2 antigens from mitotic extracts, with 20 molecular weights similar, although not identical, to those present in human cells. Again, this interaction between Pin1 and *Xenopus* MPM-2 antigens was specific as it was not detected if the precipitation was performed with control GST glutathione beads. These results demonstrate that Pin1 also interacts with a subset of conserved mitosis-specific phosphoproteins in *Xenopus*.

25 Example 13: Mutations in the Binding Pocket Abolish the Ability of Pin1 to Interact with Most Mitotic Phosphoproteins

The above results demonstrate that Pin1 directly binds numerous conserved mitotic phosphoproteins in a mitosis-dependent manner. To insure that this interaction is highly specific for Pin1, site-specific mutations were introduced into

Pin1. A high resolution X-ray structural and preliminary functional analysis of Pin1 suggest that a basic cluster consisting of Lys63, Arg68, and Arg69 is likely to be coordinate the putative phosphate group in the substrate. Ala substitutions at these residues (Pin1^{R68,69A}) should cause a reduction in the ability to bind phosphorylated residues N-terminal to the target Pro residue in the substrate. In addition, His59 has been shown to have an intimate contact with the cyclic side chain of the catalyzing Pro residue. An Ala substitution at His-59 of Pin1 (Pin1^{H59A}) should therefore disrupt the interaction between Pin1 and the substrate Pro residue.

The mutant proteins were expressed and purified as GST fusion proteins, and both their PPIase activity and their ability to bind mitotic phosphoproteins were determined. PPIase activity was assayed with two peptide substrates: AEPF, which has an acidic residue at the position N-terminal to the catalytic Pro residue, and AAPF, which does not. Pin1 had a strong preference for the AEPF substrate. The PPIase activity of Pin1^{R68,69A} was reduced more than 90% against AEPF, whereas the reduction was very small against AAPF. Moreover, Pin1^{R68,69A} had little preference for either substrate. These results confirm that residues Arg68 and Arg69 are critical for promoting strong selection for a negatively charged residue at the position N-terminal to the substrate Pro residue. The PPIase activity of Pin1^{H59A} was barely detectable against either peptide substrate, confirming the importance of His59 in Pin1 substrate binding and/or catalysis.

To determine if the Pin1 mutants interact with mitotic phosphoproteins, GST-Pin1, -Pin1^{R68,69A} and -Pin1^{H59A} fusion proteins were incubated with interphase or mitotic HeLa cell extracts and associated proteins subjected to MPM-2 immunoblotting analysis. Pin1 specifically interacted with MPM-2 antigens in two independently prepared mitotic extracts, but the binding activity of both Pin1^{R68,69A} and Pin1^{H59A} was significantly reduced compared to the wild-type protein. A few proteins including the most strongly reacting p55 band could still be recognized. The two Pin1 mutants also failed to bind most mitotic phosphoproteins from *Xenopus* extracts. Thus, mutating the residues that are implicated in binding either the substrate's putative phosphate group or the substrate's Pro residue abolish the

ability of Pin1 to bind MPM-2 antigens. This suggests that Pin1 must recognize both the Phospho Ser/Thr and the Pro residues to bind MPM-2 antigens. These results also demonstrate that mitotic phosphoproteins specifically interact with active site residues of Pin1

5 Example 14: Identification of Several Mitotic Regulators as Pin1 Targets

Several known mitotic regulators such as cyclin B, Cdc25, Myt1, Plk1 and Cdc27 are phosphorylated at mitosis. To identify at least a few of the many Pin1 binding proteins. Pin1-binding proteins were precipitated from HeLa cells, or Xenopus extracts and probed with antibodies specific for known mitotic phosphoproteins. As shown previously, levels of Plk1 and cyclin B1 increased at mitosis, whereas similar amounts of Cdc25C were present in interphase and mitotic HeLa cells. Moreover, a significant fraction of Cdc25C, Plk1, Myt1, Cdc27 and PTP-1B became hyperphosphorylated during mitosis and exhibited a shift in electrophoretic mobility by SDS-PAGE. Although cyclin B1 and PTP-1B were not precipitated by Pin1 in either interphase or mitotic extracts, Pin1 bound selectively only to the mitotically hyperphosphorylated form of Cdc25C, Plk1, Myt1 and Cdc27. Furthermore, neither mutant Pin1^{R68,69A} nor Pin1^{I159A} interacted with Cdc25 or Cdc27, indicating that the residues that are implicated in binding either the substrate's putative phosphate group or the substrate's Pro residue are necessary for 10 Pin1 to bind Cdc25 and Cdc27. Similarly, only the mitotic, but not the interphase form of Xenopus Cdc27 was precipitated by Pin1. Moreover, pretreatment of the mitotic extract with calf intestine phosphatase (CIP) completely dephosphorylated Cdc27 and abolished the interaction between Pin1 and Cdc27, demonstrating a phosphorylation-dependent interaction. These results indicate that the interaction 15 between Pin1 and Cdc25 or Cdc27 is likely to be mediated by a Phospho.Ser/Thr-Pro motif.

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To gain a sense of the generality of the interaction between Pin1 and mitotic phosphoproteins and to confirm the Pin1 interaction with target proteins is indeed mediated by phosphorylation, the ability of Pin1 to bind other known mitotic

phosphoproteins and a set of mitotic phosphoproteins identified by a systematic phosphoprotein screen (Stukenberg *et al.*, *Curr Biol* 7:338-348 (1997). Proteins synthesized in vitro were phosphorylated in a cell cycle specific manner by incubating them in either *Xenopus* interphase or mitotic extracts. These labeled 5 protein were subsequently incubated with GST-Pin1 beads that were extensively washed and the bound proteins analyzed by SDS-PAGE. To validate this method, Cdc25 was first tested. Again, the mitotically phosphorylated form of in vitro translated Cdc25 could be precipitated by GST-Pin1 beads. However, Cdc25 was not recognized by Pin1 if it was incubated in interphase extracts. Moreover, Pin1 10 did not interact with Cdc25 if the mitotically phosphorylated Cdc25 was treated with phosphatase prior to the GST-Pin1 incubation. These results demonstrate that this method can be used to detect mitosis-specific and phosphorylation-dependent interactions between Pin1 and phosphoproteins. Out of the 13 mitotic phosphoproteins examined, Pin1 bound 10 in a mitosis and phosphorylation- 15 dependent manner (summarized in Table 1), including Wee1, MP75 and MP110, MP75 and MP110 are *Xenopus* proteins related to microtubule-associated protein E-MAP-115 and the fission yeast G2 transcription factor Cdc5, respectively. These results indicate that Pin1 may target many mitosis-specific phosphoproteins.

Example 15: Pin1 Blocks Cell Cycle Progression in *Xenopus* Embryos and Entry 20 into Mitosis in *Xenopus* Extracts

Since Pin1 is conserved from yeast to humans, it is likely that Pin1 exists in *Xenopus*. To confirm this, *Xenopus* egg extracts were immunoblotted with two separate anti-human Pin1 antisera. Both antibodies, but not their respective preimmune sera, specifically recognized a band which comigrated with human Pin1 25 at 18 kDa, indicating that Pin1 is present in *Xenopus*.

Overexpression of Pin1 has been shown to inhibit cell cycle progression in both yeast and HeLa cells. To examine whether increasing the concentration of Pin1 has similar biological effects in *Xenopus*, Pin1 or Pin1 mutants were injected into one cell of 2 cell stage embryos and allowed the embryos to develop for 3 h (about 5

divisions). Wild-type Pin1 injected cells failed to cleave or cleaved slowly when compared to the cells in the uninjected side. A similar concentration (4 μ M final) of either Pin1 mutant had little effect on the cell cycle. In a separate experiment Pin1 blocked cleavage of the injected cells in a concentration-dependent manner, and at a 5 concentration approximately 20 fold above the estimated endogenous levels (10 μ M), completely inhibited the cell cycle (Figure 1). In contrast, higher concentrations of the mutant proteins were needed to block the cell cycle (Figure 1). Injection of control BSA had no obvious effect on cell cycle progression. These 10 results suggest that Pin1 must bind mitotic phosphoproteins in order to block cell cycle progression. To determine the nature of the cleavage block in *Xenopus*, GST-Pin1 was added to *Xenopus* egg extracts that had been arrested in second meiotic metaphase due to the activity of cytostatic factor. These extracts are arrested in mitosis (meiosis II) and reenter the cell cycle in response to the addition of Ca^{2+} . Extracts containing demembranated sperm to monitor nuclear morphology and 15 rhodamine-tubulin to monitor microtubule spindle assembly, were activated with Ca^{2+} . Pin1 was added after the extracts had entered interphase (15 min after the addition of Ca^{2+}), and the subsequent entry of the extracts into mitosis was followed by nuclear morphology and Cdc2 kinase activity using histone H1 as a substrate. Addition of either 10 or 40 μ M Pin1, approximately 20 or 80 fold higher than 20 endogenous levels, completely blocked entry into mitosis as detected by the persistence of interphase nuclei and low Cdc2 kinase activity. In contrast, the same extracts containing 40 μ M of either BSA or the mutant Pin1 proteins entered mitosis by 70 to 80 min as detected by nuclear envelope breakdown, spindle formation and high histone H1 kinase activity. Thus, as was shown previously in HeLa cells, 25 increasing the Pin1 concentration causes a cell cycle block in G2. More importantly, Pin1 must bind mitotic phosphoproteins to elicit this phenotype.

Example 16: Pin1 Binds and Inhibits Mitotically Phosphorylated Cdc25

The above results indicate that overexpression of Pin1 inhibits mitotic entry in *Xenopus*, as is the case in HeLa cells and yeast. Entry into mitosis is regulated by

dephosphorylation of Cdc2 by the phosphatase Cdc25, and Cdc25 is activated by mitosis-specific phosphorylation at the MPM-2 epitope at the G2/M transition.

Earlier results indicated that it is the mitotically phosphorylated form of Cdc25 that interacts with Pin1 in vitro. Therefore, it is conceivable that the inhibitory effects of

- 5 Pin1 on entry into mitosis could at least partially explained through inhibition of Cdc25 activity.

To test this possibility, it was determined whether Pin1 interacts with Cdc25 in vivo and if so, whether this interaction is cell cycle regulated. *Xenopus* eggs were collected at various times after fertilization and subjected to immunoprecipitation 10 using anti-*Xenopus* Cdc25 antibodies as well as histone H1 kinase assay to monitor cell cycle progression. When the resulting Cdc25 immunoprecipitates were immunoblotted with anti-Pin1 antibodies, we found that endogenous Pin1 was precipitated by anti-Cdc25 antibodies. Furthermore, this interaction between Pin1 and Cdc25 was cell cycle-regulated, significantly increased just prior to mitosis.

15 Similar results were also obtained using synchronized HeLa cells using anti-human Cdc25C. Unfortunately, we were not able to detect Cdc25 in anti-Pin1 immunoprecipitates, probably because the amount of Cdc25 precipitated is below the detection of the Cdc25 antibodies. It is worth of pointing out that the percentage of coimmunoprecipitable Pin1 and phosphorylated Cdc25 is not high. This might 20 be expected because the complex might not be stable to the stringent immunoprecipitation conditions. the amount of Cdc25 phosphorylated on Pin1-binding sites might be low at this point, and/or the complex might have a high off rate, since the phosphorylated Cdc25 is a substrate of Pin1. Nevertheless, these 25 results suggest that Pin1 is associated with Cdc25 at a time when Cdc25 is partially phosphorylated and yet its activity is low.

Since the interaction between Pin1 and Cdc25 is mediated by phosphorylation of Cdc25, it was determined whether Pin1 interacts with Cdc25 on important phosphorylation sites. At entry into mitosis, Cdc25 is phosphorylated at multiple Thr/Ser-Pro (Peng, C. Y. Graves, P. R., Thoma, R. S. Wu, Z. Shaw, A. S. and 30 Piwnica-Worms, H. *Science*, 277:1501-1505 (1997)).

Izumi and Maller (Izumi, T. and J.L. Maller, *Mol Biol Cell* 4:1337-1350 (1993)) have shown that the triple mutation of conserved Thr48, Thr67 and Thr138 (T3 Cdc25), and the quintuple mutation of the three Thr residues plus Ser205 and Ser285 (T3S2 Cdc25) prevent most of the shift in electrophoretic mobility of Cdc25 after 5 incubation with mitotic extracts. When they measured the ability of the Cdc25 mutants to activate Cdc2 in the Cdc25-depleted oocyte extracts and to initiate mitotic entry in oocyte extracts, the activities of T3 and T3S2 mutants were reduced about 70% and 90%, respectively (Izumi and Maller, 1993). These results indicate that these Thr/Ser residues are essential for the Cdc25 function. We examined the 10 ability of Pin1 to bind the T3 and T3S2 Cdc25 mutants. As shown previously (Izumi and Maller, 1993), the T3 and T3S2 Cdc25 mutants failed to undergo the mobility shift after incubation with mitotic extracts. Although Pin1 strongly bound mitotically phosphorylated form of Cdc25, Pin1 almost (T3) or completely (T3S2) failed to bind the Cdc25 mutants which were incubated with either interphase or 15 mitotic extracts. Although further experiments are required to pinpoint which phosphorylation site(s) play(s) the major role in mediating the Pin1 and Cdc25 interaction, these results show that Pin1 interacts with the phosphorylation sites on Cdc25 that are essential for its mitotic activation.

The above results indicate that Pin1 interacts with Cdc25 both in vitro and in 20 vivo. Therefore, we tested whether Pin1 could affect the physiological activity of Cdc25, which is to dephosphorylate and activate the cyclin B/Cdc2 complex. To generate the mitotically phosphorylated form of Cdc25, GST-Cdc25 was incubated in Xenopus mitotic extracts, affinity purified on glutathione agarose beads and eluted. This mitotic Cdc25 was at least 9 fold more active than GST-Cdc25 purified 25 in parallel from interphase extracts (data not shown). This mitotic GST-Cdc25 activated cyclin B/Cdc2 complex that was kept inactive due to inhibitory phosphorylations on Tyr15 and Thr14. If Pin1 (0.67 μ M) was included in the assay at amounts approximately stoichiometric to mitotic Cdc25 (1 μ M), mitotic Cdc25 failed to activate the Cdc2 complex. In contrast, neither the mutant Pin1^{R68,69A} at the 30 same concentration (0.67 μ M), or BSA at a 25 fold higher concentration (15 μ M)

had a significant inhibitory effect on Cdc25 activity (Figure 3A). Five fold higher concentrations of Pin1^{R68,69A} could partially inhibit mitotic Cdc25 activity (Figure 3B), a result which is consistent with the requirement for higher concentrations of this mutant protein to arrest the *Xenopus* cell cycle. To rule out the possibility that

- 5 Pin1 could directly inhibit the cyclin B/Cdc2 complex itself, we examined the effect of Pin1 and its mutants on the activity of dephosphorylated-active cyclin B/Cdc2 under same conditions. Neither Pin1 nor the Pin1 mutant had any effect on Cdc2 activity (Figure 3C). Taken together, these results indicate that Pin1 could inhibit premature mitotic activation of Cdc25 by interacting with the phosphorylation sites
10 on Cdc25 that are essential for its activation. This offers one explanation for the ability of Pin1 to inhibit mitotic entry.

Example 17: Screening of Peptide Libraries

The oriented peptide library approach (Z. Songyang et al. *Cell*, 72:767 (1993) was used to screen for optimal peptides. All amino acids except C were incorporated
15 at equimolar amounts in each degenerate position, yielding a total theoretical degeneracy for both libraries of $19^6 = 4.7 \times 10^7$ distinct peptide sequences. Pin1-GST beads and MPM2 antibody bound to protein-G beads were incubated with the peptide library mixtures and washed extensively. Bound peptides were eluted with 30% acetic acid and sequenced.

- 20 The chromogenic oligopeptides were synthesized (A. Bernhardt, M. Drewello, & M. Schutkowski, *Int. J. Peptide Protein Res.* 50:143 (1997)) and confirmed by NMR. Standard peptides were purchased from Bachem. PPIase activity were assayed and the bimolecular rate constants k_{cat}/K_m were calculated according to the equation $k_{cat}/K_m = (k_{obs} - k_u)/[PPIase]$, where k_u is the first-order rate constant for spontaneous
25 *cis/trans* isomerization and k_{obs} is the pseudo-first-order rate constant for *cis/trans* isomerization in the presence of PPIase, as described in G. Fischer, H. Bang, C. Mech, *Biomed. Biochim. Acta* 43:1101 (1984); J. L. Kofron et al. *Biochemistry* 30:6127 (1991). Affinity of Pin1 for peptides was measured as described in Schutkowski, M., Wöllner, S., & Fischer, G. *Biochemistry* 34:13016, (1995).

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EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

CLAIMS

What is claimed is:

1. An inhibitor of a phosphoserine- or phosphothreonine-proline specific peptidyl-prolyl isomerase comprising a molecule that mimics the structure and conformation of the pSer/pThr-Pro peptide moiety of the isomerase substrate when the substrate is bound into the active site of the isomerase.
2. The inhibitor of Claim 1 wherein the structure surrounding the mimic moiety is flanked on one side by hydrophobic residues and on the other side by hydrophobic or positively charged groups wherein the groups contact the active site of the isomerase.
3. An inhibitor of a phosphoserine- or phosphothreonine-proline specific peptidyl-prolyl isomerase comprising a protein, peptide or peptide mimetic comprising xSer/ThrY wherein x is a negatively charged tetra or pentavalent moiety and Y is a Pro or a Pro analog.
4. The inhibitor of Claim 3 wherein x is selected from the group consisting of phosphate, sulfonate, boronate, phosphonate and sulfonyl amide.
5. The inhibitor of Claim 3 wherein the proline analog is a nitrogen-containing ring structure selected from the group consisting of imidazole, pyrrole, tropolone, benzene, camphor and heterocyclic aromatic and non-aromatic ring structures.
6. The inhibitor of Claim 3 wherein the K_i of the inhibitor is ten micromolar or less.

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7. The inhibitor of Claim 3 wherein xSer/Thr-Y is flanked on one side by hydrophobic residues and on the other side by hydrophobic residues or positively charged residues.
8. The compound of Claim 3 wherein the protein, peptide or peptide mimetic is linear or cyclic.
5
9. A method of inhibiting cell growth comprising inhibiting a mitotic peptidyl-prolyl isomerase in the cell comprising contacting the cell with an effective amount of the inhibitor of Claim 3.
10. The method of Claim 9 wherein the mitotic peptidyl-prolyl isomerase is Pin1.
11. The method of Claim 9 wherein the cell is in an individual.
10
12. The method of Claim 9 wherein the cell growth results from a hyperplastic or neoplastic disorder.
13. The method of Claim 9 wherein the cells are eukaryotic cells.
14. The method of Claim 9 wherein the cells are selected from the group consisting of: mammalian cells, yeast cells and fungal cells.
15
15. A composition comprising an inhibitor of Claim 3 and a pharmaceutically-acceptable carrier.
16. A compound that inhibits a phosphoserine- or phospho threonine-proline specific peptidyl-prolyl isomerase comprising a protein, peptide and/or a peptide mimetic wherein said protein, peptide or peptide mimetic has a core sequence of XXXpSer-ProXXX, wherein X is any L-amino acid, or D-amino acid.
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17. The compound of Claim 16 wherein the protein, peptide or peptide mimetic is at least eight amino acid residues in length and comprises the sequence $X_1X_2X_3pS-PX_4X_5X_6$ wherein each residue can be independently selected as follows X_1 is W, Y or F; X_2 is F or I; X_3 is Y, R, F or W; X_4 is R, F, Y or W; X_5 is L or I and X_6 is any amino acid.
5
18. The compound of Claim 16 wherein the inhibitor has a K_i of ten micromolar or less.
19. A peptide inhibitor of a phosphoserine- or phosphothreonine-proline-specific peptide prolyl isomerase comprising Trp-Phen-Tyr-pSer-Pro-Arg.
- 10 20. A library of peptides that comprises a mixture of substantially equimolar amounts of peptides comprising the sequence $NH_2-MAXXXpSXXXAKK$, wherein for each peptide X is any amino acid.
21. A library of compounds that comprises a mixture of substantially equimolar amounts of peptides comprising the sequence $X_1X_2X_3pS-PX_4X_5X_6$, wherein for each peptide X is any amino acid.
15
22. A method of identifying a phosphoserine- or phosphothreonine-specific peptidyl prolyl isomerase inhibitor comprising the steps of:
 - a) providing a library of compounds that comprises a mixture of substantially equimolar amounts of peptides comprising the sequence $X_1X_2X_3pS-PX_4X_5X_6$, wherein for each peptide X is any amino acid;
20
 - b) contacting the library of a) with the peptidyl prolyl isomerase of interest under binding conditions for time sufficient for the isomerase to bind to the peptides;
 - c) determining the amino acid sequences of peptides bound to the isomerase of interest;
25

- d) synthesizing the peptides of c); and
- e) determining the K_i of the peptide wherein a K_i of ten micromolar or less indicates that the peptide is suitable for use as an inhibitor of the isomerase of interest.

5 23. A method of identifying a phosphoserine- or phosphothreonine-specific peptidyl prolyl isomerase inhibitor comprising the steps of:

- a) providing the peptidyl prolyl isomerase of interest;
- b) mixing the isomerase of interest with:
 - i) a candidate inhibitor molecule and
 - ii) the substrate of the isomerase of interestto form an admixture of the isomerase of interest, candidate molecule and substrate;
- c) maintaining the admixture of b) under conditions sufficient for the isomerase of interest to catalyze the *cis/trans* isomerization of the substrate; and
- d) determining the K_i of the candidate molecule, wherein a K_i of 10 micromolar or less is indicative of an inhibitor of the peptidyl prolyl isomerase of interest.

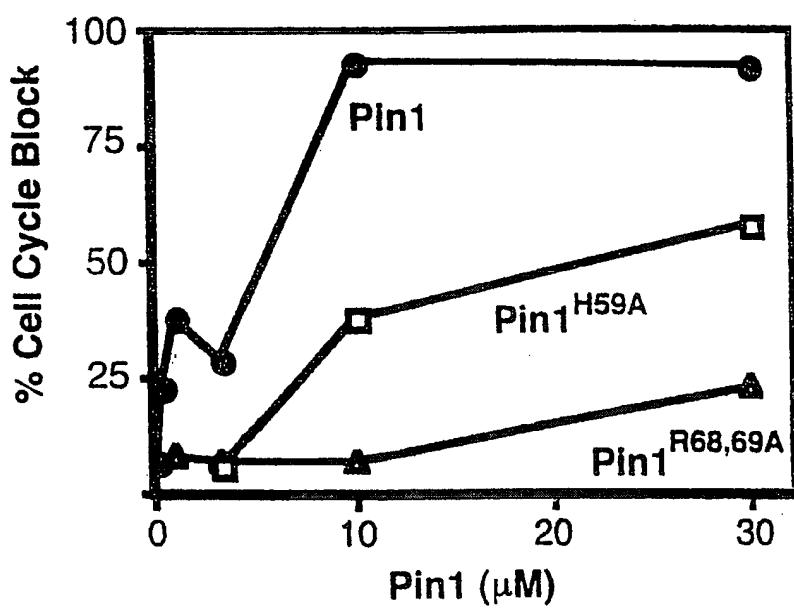


FIG 1

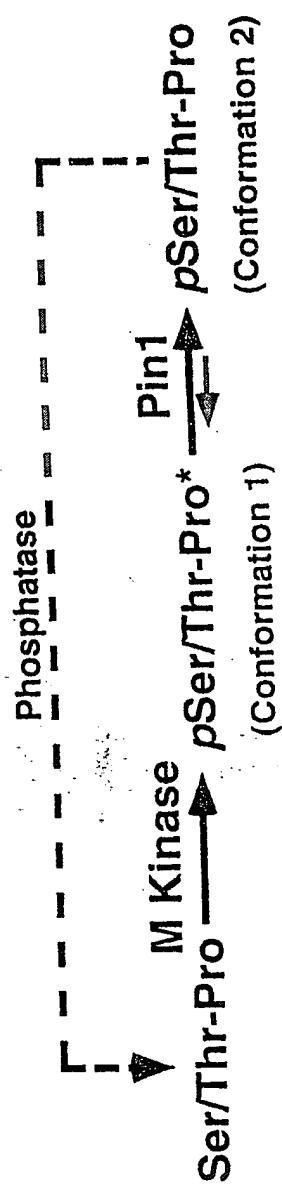


FIG. 2

Fig. 3A

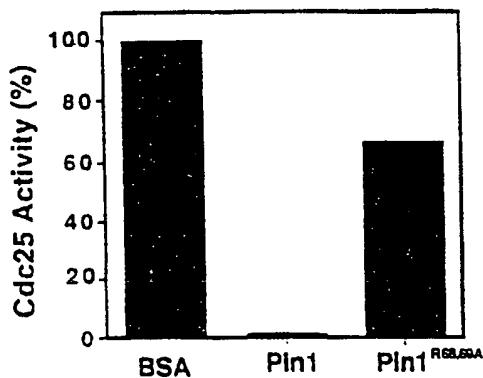


Fig. 3B

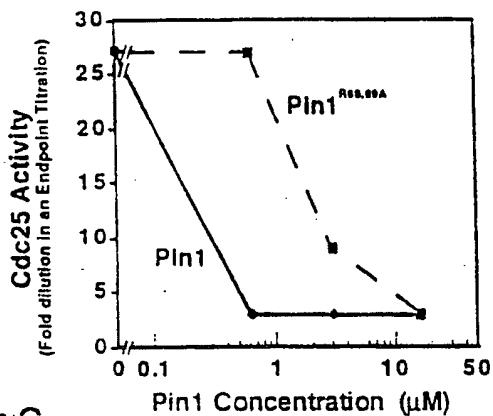
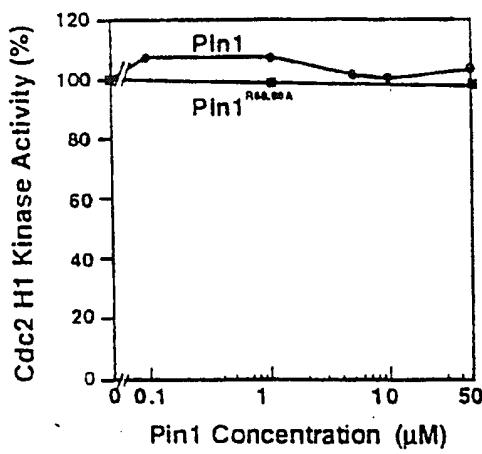


Fig. 3C



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/18862

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C07K14/00 C07K1/04 C07K1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>CHEMICAL ABSTRACTS, vol. 126, no. 26, 30 June 1997 Columbus, Ohio, US; abstract no. 340373, H H LUDWIG ET AL.: "Synthesis and kinetic properties of various side chain modified peptide derivatives as effectors of prolyl endopeptidase" XP002090550 & PERSP. PROT. ENG. '96, 5TH, PAPER NO. 10,1996, pages 1-5, Biodigm, Bingham, UK see abstract</p> <p>---</p> <p style="text-align: center;">-/-</p>	1,16

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

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Date of mailing of the international search report

20 January 1999

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 451992 Derwent Publications Ltd., London, GB; AN 92-369428 XP002090552 "New phospho peptide useful antigen preparation antibody pair helical filament present brain patient disease" & JP 910 146 477 A (MITSUBISHI KASEI CORP.), 25 February 1991 see abstract ---	1,16
X	R RANGANATHAN ET AL.: "Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent " CELL, vol. 89, 13 June 1997, pages 875-886, XP002090538 NA US see the whole document ---	1,16
X	K P LU ET AL.: "A human peptidyl-prolyl isomerase essential for regulation of mitosis" NATURE., vol. 380, no. 6574, 11 April 1996, pages 544-547, XP002090539 LONDON GB see the whole document ---	1,16
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X	G I WELSH ETAL.: "Peptide substrates suitable for assaying glycogen synthase kinase-3 in crude cell extracts" ANALYTICAL BIOCHEMISTRY., vol. 244, 1997, pages 16-21, XP002090541 NEW YORK US see column 17, line 38 - line 41 ---	1,16
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M TSUKAMOTO ET AL.: "Improved protective groups for phosphate of O-phosphoserine useful for the solid-phase peptide synthesis" TETRAHEDRON LETTERS, vol. 32, no. 48, 25 November 1991, pages 7083-7086, XP002090542 OXFORD GB see peptide 7, scheme 2 ---	1,16
X	G SHAPIRO ET AL.: "FMOC solid phase synthesis of serine phosphopeptides via selective protection of serine and on resin phosphorylation" TETRAHEDRON LETTERS, vol. 35, no. 6, 7 February 1994, pages 869-872, XP002090543 OXFORD GB see figure 1 ---	1,16
X	R A HODGES & J A SMITH, EDS.: "Peptides, structure, chemistry and biology" 1994, ESCOM, LEIDEN, THE NETHERLANDS XP002090549 206510 see page 799 - page 801 ---	1,16
X	K A RESING ET AL.: "Mass spectrometric analysis of 21 phosphorylation sites in the internal repeat of rat profilaggrin, precursor of an intermediate filament associated protein" BIOCHEMISTRY., vol. 34, no. 29, 25 July 1995, pages 9477-9487, XP002090544 EASTON, PA US see figures 3,4,8 ---	1,16
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X	C J FIOL ET AL.: "Ordered multisite protein phosphorylation" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 265, no. 11, 15 April 1990, pages 6061-6065, XP002090546 MD US see table IV ---	1,16

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International Application No

PCT/US 98/18862

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	L OTVOS JR. ET AL.: "Solid-phase synthesis of peptides" INTERNATIONAL JOURNAL OF PEPTIDE AND PROTEIN RESEARCH., vol. 34, no. 2, July 1989, pages 129-133, XP000027928 COPENHAGEN DK see page 130, line 12 ----	1,16
P,X	M B YAFFE ET AL.: "The structural basis for 14-3-3: phosphopeptide binding specificity" CELL, vol. 91, no. 7, 26 December 1997, pages 961-971, XP002090547 NA US see the whole document ----	1-23
P,X	M B YAFFE ET AL.: "Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulation mechanism" SCIENCE, vol. 278, 12 December 1997, pages 1957-1960, XP002090548 LANCASTER, PA US see the whole document ----	1-23
A	CHEMICAL ABSTRACTS, vol. 118, no. 19, 10 May 1993 Columbus, Ohio, US; abstract no. 186454, R A LINDBERG ET AL.: "Characterization of a human protein threonine kinase isolated by screening an expression library with antibodies to phosphotyrosine " XP002090551 & ONCOGENE, vol. 8, no. 2, 1993, pages 351-359, see abstract -----	19-23